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Prostate cancer cells enhance IL-15-mediated expansion of NK cells

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Running title: Prostate cancer cells enhance IL-15-mediated expansion of NK cells

Abstract

Objectives:

To identify cytokines that can activate and expand NK cells in the presence of prostate cancer cells in order to determine whether these agents may be useful in future intratumoral administration in pre-clinical and clinical prostate cancer trials.

Materials and Methods

Lymphocytes isolated from normal donor blood were set up in co-cultures with either cancer or non-cancerous prostate cell lines, together with each of the cytokines IL-2, IL-12, IL-15, Interferon gamma (IFN- γ) or IL-21 for a period of 7 days. Then, expansion of NK cells, NKT cells and CD8 T cells was measured by flow cytometry and compared with the expansion of the same cells in the absence of prostate cells. The cytotoxic activity of NK cells as measured by perforin and tumour cell killing was also assessed. NK cell receptors and their corresponding ligands on prostate tumour cells were analysed to determine whether any of these were modulated by co-culture. The role of the tumour secreted heat shock proteins HSP90 and HSP70 in expansion of NK cells in the co-cultures was also investigated due to their effects on NK and CD8 T cell activation.

Results

We show that only IL-15, among a panel of cytokines known to cause NK cell activation and expansion, can actively induce expansion of NK, NKT and CD8 T cells in the presence of prostate cancer cell lines. Furthermore, the expansion of NK cells is far greater (up to 50% greater) in the presence of the cancer cells (LNCaP, PC3) than when lymphocytes are incubated alone. In contrast, non-cancerous cell lines (PNT2 and WPMY-1) do not exert any expansion of NK cells. The cytolytic activity of the NK cells, as measured by perforin, CD107a and killing of tumour cells is also greatest in co-cultures with IL-15. Examination of NK cell receptors shows that NKG2D is upregulated to a greater degree in the presence of prostate cancer cells, compared with the upregulation with IL-15 in lymphocytes alone. However, blocking of NKG2D does not inhibit the enhanced expansion of NK cells in the presence of tumour cells.

Conclusions

IL-15 is the only cytokine among a panel of NK cell activating cytokines that can stimulate expansion of NK cells in the presence of prostate cancer cells. Therefore IL-15 may be a good candidate for novel future intratumoral therapy of the disease.

Introduction

Immunosuppression exerted by the tumour microenvironment is a major challenge in tumour immunotherapy. In particular, the prostate cancer microenvironment exerts considerable immunosuppressive effects as shown by the presence of anergic NK and T cells, and regulatory T cell populations with the milieu and is known as an immunologically “cold”

tumour type [1, 2]. However, the finding that lymphocytes infiltrate the cancerous prostate, combined with the knowledge of prostatic antigens such as prostate-specific acid phosphatase (PAP), prostate-specific membrane antigen (PSMA), and NKX3.1 present on cancer cells in higher amounts than on normal cells, [3-5], led to the advent of prostate cancer vaccines, such as Provenge™ [6], that created new interest in the use of immunotherapies against prostate cancer. However, in prostate cancer, no significant clinical responses have been observed with well-known checkpoint immunotherapies such as Ipilimumab, anti-PDL1 or anti-PD-1[7, 8], although a minority of patients (5%) treated with the latter agents are responsive due to defects in genomic instability or DNA mismatch repair genes causing increases in tumour mutational burden and neoantigen expression[9-11]. The regulation of expression of these checkpoint proteins is also still not understood. As novel checkpoints either on tumour cells or the surrounding cells of the milieu are being discovered regularly, it may be impossible to target all of these as a therapeutic approach to block immunosuppression in the tumour lesion.

Therefore, agents that can drive the activation and proliferation of NK and other effector cell populations within the cancer microenvironment may give a more favorable response than multiple antibodies directed at single immunomodulatory checkpoint targets.

One of the most promising cytokines in cancer immunotherapy is Interleukin 15 (IL-15). IL-15 is a pleiotropic cytokine with two receptors common to IL-2 (IL2R β (CD122) and IL2R γ (CD132)) in addition to its individual receptor, the IL-15 alpha receptor (IL-15R α (CD215) [12]. These receptors can be found on haematopoietic cell types including NK cells, monocytes, and T cells. IL-15 is secreted from cell types including dendritic cells, monocytes and epithelial cells and activates both NK and CD8 T cells among a number of immune effector cells; therefore it has been identified as an agent that can induce anti-tumour immunity alongside IL-2 [13]. NK cells and CD8 T cells, in particular, memory T cells,

proliferate in the presence of IL-15 both in-vitro and when adoptively transferred into animals[14]. IL-15 has been shown to effectively reduce prostate tumour volume in TRAMP-C2 challenged C57-BL6 mice[15]. In addition, a number of studies involving in-vivo murine tumour challenge models using IL-15 transfected tumour cell lines, including prostate cancer cell lines, have shown that these tumours exhibit greatly reduced growth and a marked infiltration of NK cells and macrophages[16-18]. This is due to the fact that IL-15 can upregulate the expression of the receptors CXCL9 and CXCL10 on epithelial cells, which leads to recruitment of NK cells to these cells through CXCR3[19]. Moreover, heat shock proteins such as HSP70, that are secreted constitutively by many tumour cell lines, but not by normal epithelial cells have been reported to increase IL-15 or IL-2 mediated expansion of immune cells including NK cells [20-23].

However, IL-15 and other therapeutic cytokines used in anti-cancer therapy such as IL-2, IL-12 and IL-21 have not been studied in the context of their effects on immune effector cells exposed to prostate cancer cells. We therefore examined the effects of IL-15 and several other TH1 promoting therapeutic cytokines on NK and CD8 T cell expansion and cytotoxic function in the absence or presence of a number of prostate cancer and non-cancerous prostate cell lines using an in-vitro co-culture model. The effects of IL-15 on the expression of a number of NK cell receptors and their ligands were also examined in the co-cultures. In addition, we also assessed whether IL-15 in co-cultures of lymphocytes and cancer cells was increasing the production of HSP70 and HSP90 heat shock proteins expressed in prostate cells.

Materials and Methods:

Cell lines

The cell lines LNCaP, PC-3, PNT2, WPMY-1 and TRAMP-C1 were obtained in the last 3 years from the ATCC (LGC standards, UK). LNCaP and PC3 are human metastatic prostate epithelial carcinomas. TRAMP-C1 is a tumourigenic mouse prostate adenocarcinoma cell line obtained from the transgenic adenocarcinoma of the mouse prostate (TRAMP) immunocompetent C57/BL6 mouse prostate model. PNT2 is an epithelial prostate cell line derived from normal prostate epithelial cells that has been immortalized with SV40. WPMY-1 is a myofibroblast stromal cell line also immortalized by SV40. WPMI and PNT2 are termed “non-cancerous cells” in this investigation.

LNCaP, PC-3, and PNT2 cell lines were cultured in RPMI medium containing, 2mM glutamine, 100 units/ml of penicillin and streptomycin (Sigma Aldrich UK) and 10% foetal bovine serum (Life Technologies UK) (RPMI complete medium). WPMY-1 cells were cultured in DMEM medium supplemented as above. TRAMP-C1 cells were cultured in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, supplemented with 0.005 mg/ml bovine insulin and 10 nM dehydroisoandrosterone, 5% Nu-Serum IV (all components from Sigma Aldrich, UK), and 5% fetal bovine serum.

Isolation of PBMCs, and depletion of CD8 T cells or CD56+ cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using anonymized leukodepletion cones (Blood Transfusion Service, NHS Blood and Transplantation). Blood from the leukodepletion cones was diluted by 50% in Hanks buffered saline solution (Sigma Aldrich UK) and layered at a 1:1 ratio on Histopaque 1077 (Sigma

Aldrich UK) using 50 ml Falcon tubes. The PBMCs were then prepared from the buffy coats as previously described [24]. To prepare non-adherent PBMCs for the co-cultures of lymphocytes and prostate cancer cells, PBMCs were added to 175cm² culture flasks at 3 x 10⁶ cells/ml in RPMI complete medium and left for 3 h to adhere to the plastic. After 3 h, the non-adherent cells were harvested for use in the co-cultures.

PBMCs were depleted of CD8 T cells or CD56⁺ cells using a Miltenyi isolation kit as per manufacturer's instructions. Depletions were successful with a removal of at least 99% of the CD56⁺ or CD8⁺ populations from these lymphocytes.

Lymphocyte: Cancer cell Co-Culture assay

Non-adherent PBMCs were co-cultured with cancer or non-cancerous prostate cell lines for 7 days at a ratio of 8:1 in 24-well plates using a working concentration of 8 x 10⁵ PBMCs to 1 x 10⁵ prostate cells. The 8:1 ratio had been previously shown to give the optimal NK expansion and NK cell cytotoxic activity following co-cultures carried out at 1:1, 2:1, 4:1, and 20:1 of lymphocytes to prostate cells[25]. To these cultures, cytokines were added, either IL-2, IL-12, IL-15, INF- γ , or IL-12 at a dose of 25 ng/ml (all cytokines from Peprotech, UK). The 25 ng/ml dose was used as this gives maximal secretion of IFN- γ from NK cells (ED50s of the cytokines range from 0.1-2 ng/ml according to the Peprotech datasheets). Prostate cells were irradiated with 4 grays using a gamma irradiator to inhibit their proliferation during the 7 day co-culture with minimal effects on their viability [26]. In some experiments, a 0.4 μ M transwell filter was included, (referred to as an insert), between the lymphocytes and the prostate cells, to determine whether proliferation of effector cells occurred in the absence of contact with prostate cells.

For co-cultures using murine cells, splenocytes were isolated from the spleens of C57BL6 mice (aged 10-12 weeks), treated with red cell lysis buffer to remove red blood cells and then MHC class II depleted (kit from Miltenyi UK) to remove monocytes and macrophages. The resulting lymphocytes were co-cultured at an 8:1 ratio using the murine forms of the above cytokines at 25 ng/ml each using a working concentration of 8×10^5 splenocytes to 1×10^5 irradiated TRAMP-C1 tumour cells for 9 days.

For all cultures, medium, and cytokines in the wells was replenished at day 3 to prevent nutrient loss to the cells. Lymphocyte viability in the presence of the cytokines was always >90%.

Analysis of NK, NKT and T cell expansion, cytotoxic activity, and killing of tumour cells

NK, NKT, T cells and regulatory T cells (Tregs) populations within the PBMCs were measured using a BD FACsCalibur™ (BD Biosciences UK) with fluorophore conjugated mouse anti-human anti-CD4 (clone A161A1), CTLA-4 (clone BNI3), CD25 (clone BC96) and FOXP3 (clone 206D) (Biolegend, UK) for the Treg analysis and anti-human CD56 (clone MEM-188), CD3 (clone HIT1A) (Biolegend UK) and CD8 (clone BW135/80) (Miltenyi Biotech, UK) for the NK, NKT and CD8 T cells. Standard protocols for surface flow cytometric staining were followed to detect surface markers (<https://www.biolegend.com/protocols/cell-surface-flow-cytometry-staining-protocol/4283/>), whereas an intracellular staining protocol was performed to detect FOXP3 (http://www.biolegend.com/media_assets/support_protocol/Intracellular_Staining_Protocol_041515.pdf).

The immune cell mediated killing of the human tumour cells was measured using annexin/propidium iodide (PI) staining after the 7-day co-culture, followed by flow cytometric analysis.

Murine NK, NKT cells, CD8 T cells and Tregs was measured using flow cytometry with a BD FACsCalibur and the antibodies used (Biolegend UK) were anti-mouse NK1.1 (clone PK136), CD3 (clone 17A2), and CD8 (clone 53-6.7). Killing of TRAMP-C1 cells was measured by culturing the primed MHC class II depleted splenocytes, (or NK cells or CD8 T cells purified from the MHC class II depleted splenocytes by Miltenyi isolation kits) from the 9 day assay with fresh TRAMP-C1 cells and observing killing after 24 hrs with a live/dead assay (Thermofisher UK). The cytotoxic activity of the NK, NKT and CD8 T cells in the human co-cultures was assessed using antibodies to perforin (clone dG9), and CD107a (clone H4A3) (Biolegend UK). In these experiments, cells were either fixed and permeabilised prior to antibody staining as described in the Biolegend intracellular protocol (for perforin) or cells were treated with Brefeldin A for 4 hrs prior to addition of CD107a antibody to stop release of CD107a from the cell surface, and then staining with the cell surface marker protocol.

Measurement of the expression of NK receptors on NK cells and NK ligands on tumour cells

To investigate the mechanisms of the IL-15 mediated expansion of NK cells in the presence of prostate tumour cells, a panel of antibodies to inhibitory and activatory NK cell receptors was used to stain NK cells in the co-cultures that were prepared as shown above. The antibodies used were mouse anti human FITC-conjugated anti-NKG2D (1D11), anti-DNAM-1 (Clone 11A8), anti-NKP30 (Clone P30-15), anti-NKP44 (Clone 3.43), anti-NKP46 (Clone 9E2), anti-KIR2DL1 (Clone HP-MA4), anti-KIR2DL2 (Clone DX27) and anti-

KIR3DL1(Clone DX9) (all purchased from Biolegend, UK) together with PerCP-conjugated mouse anti-human CD8 (Miltenyi Biotech, UK) and PE-conjugated mouse anti-human CD3 (Biolegend UK). In addition, separate experiments were carried out using a panel of antibodies to NK receptor ligands – these were mouse anti-human MICA/B (Clone 6D4 Biorad, UK), MICA(Clone 4), ULBP-1 and Nectin 2 (polyclonal antibodies) (Biorbyt USA), HLA-ABC (W6/32, Biolegend UK), HLA- BW4 (0.L.6, US Biologicals, USA), HLA-Class I (Tu149, Life technologies, UK), and HLA-G (4H84, SantaCruz, USA).

Since we had also found that 2.5 ng/ml IL-15 gave more expansion of NK cells than 25 ng/ml IL-15[25], expression of the receptors and ligands was measured after the 7 day co-culture incubations with either PBS, low dose IL-2 (10 units/ml), high dose IL-2 (100 units/ml), IL-15 (2.5 ng/ml) or IL-15 (2.5 ng/ml) plus IL-2 (10 units/ml). Low dose IL-2 was included in order to resemble the microenvironment of prostate cancer where it is known that IL-2 is present in very small amounts and is also present in the sera of patients [27].

For selected ligands that have been shown to be shed from the tumour cell surface – i.e MICA, Nectin2 –ELISAs (Biolegend UK) were performed to measure levels of these ligands in the supernatants of PBMC – tumour cell co-cultures.

Inhibition of NKG2D in the PBMC –tumour cell co-cultures

To assess whether blocking of NKG2D could inhibit the expansion seen in the tumour cell-lymphocyte co-cultures, the 7 day co-cultures were performed with either a blocking antibody to NKG2D (Ultra-LEAF™ Purified anti-human CD314 (NKG2D) Antibody [Clone: 1D11] (Biolegend UK Ltd, London, UK) or its isotype control (Ultra-LEAF™ Purified Mouse

IgG1, κ Isotype Ctrl Antibody [Clone: MOPC-21] (Biolegend UK Ltd). The antibodies were used at 10 μ g/ml as suggested in previous papers that showed blocking of NKG2D activity in NK cells, and NKG2D mediated tumour cytotoxicity with this antibody [28, 29]. After 7 days, the co-cultures were analysed for any inhibition of expansion that had been mediated by IL-15. The antibody activity was confirmed by analysing NKG2D expression on the NK cells, which was completely inhibited (data not shown).

HSP90 and HSP70 ELISAs

ELISAs were performed for Hsp70 and 90 according to the manufacturer's instructions (Biolegend, UK) in the supernatants of the co-cultures of PNT2, WPMY-1, LNCaP and PC-3 with lymphocytes to determine whether levels were lower in the non-cancerous, compared to the tumour cells.

Results

IL-15 expands NK, NKT and CD8 T cell populations within prostate cancer –lymphocyte co-cultures

To examine the effect of prostate cells on the expansion of immune effector cells in a lymphocyte population cultured with a panel of cytokines previously shown to expand NK cells, we cocultured prostate cells (either cancer cells or non-cancerous lines) with lymphocytes from normal donors. Figure 1 shows that with all prostate cell line –lymphocyte co-cultures tested, IL-15, but not IL-2, IFN- γ , IL-12 or IL-21 gave rise to expansion of NK and NKT cells. The gating strategy to measure NK expansion (CD56⁺CD3⁻ cells) and representative dot plots are shown in Figure 1a, . Figure 1b shows that treatment with IL-15

increased NK cell numbers over fivefold on average with PC-3 and LNCaP cells (n = 6, *p<0.05 by one-way anova and Newman Keuls post-hoc tests), whereas WPMY-1 and PNT2 cell co-cultures increased NK numbers by an average of 2.1-fold, similar to the expansion seen in lymphocytes alone. The expansion of NK cells with PC-3 and LNCaP was significantly greater than with lymphocytes alone (n= 6, ** p <0.05 by one-way anova and Newman Keuls post-hoc test). Therefore, IL-15 treatment of lymphocytes with both PC-3 and LNCaP cancer cell lines, but not the non-cancerous prostate cells, can increase expansion of NK cells to a much greater extent than in lymphocytes alone. NKT cells were expanded by an average of threefold and expansion was similar in co-cultures with all prostate cells with or without tumour cell contact and occurred to the same extent in lymphocytes cultured alone treated with IL-15 (n= 6, ** p <0.05 by one-way anova and Newman Keuls post-hoc test). CD8 T cells were increased by up to 1.5-fold in lymphocytes alone, by IL-15, but expansion was less when lymphocytes were in contact with PC-3 cells, whereas no expansion was observed in PNT2 cells, compared to the control (PBS) group. Using lymphocytes cultured without prostate cells, the cytokines IL-2, and IL-12 showed a moderate expansion of NK cells, and IFN- γ and IL-12 expanded NKT cells, but again, only IL-15 expanded CD8 T cells. Tregs were only increased in lymphocytes alone treated with IL-2. IL-15 inhibited Tregs both in lymphocytes alone and in the co-cultures.

Activity of IL-15 in the prostate cancer-lymphocyte co-cultures is not prostate cell-contact dependent for NK cell expansion or CD8 expansion

To examine whether contact of lymphocytes with prostate cancer cells was required for enhanced NK cell expansion, we included conditions in the co-culture assay where lymphocytes were co-cultured with prostate cells in the presence of a transwell membrane

insert which would inhibit cell-cell contact. Figure 1b shows that in the presence of IL-15, there is no significant difference in expansion of NK, NKT or CD8 T cells when the cell lines are in contact with the non-adherent lymphocytes, compared to their expansion when there is a 0.4 μ m transwell membrane (insert).

IL-15 increases the anti-tumour cytotoxic activity of NK, NKT and CD8 T cells in the co-cultures.

The cytotoxic activities of the NK, NKT and CD8 T cells were all increased by IL-15 as measured by a significant increase in intracellular expression of perforin (Figure 2b) and killing of the tumour cells measured with Annexin/PI staining (Figure 2a) (n= 6 experiments, * p<0.05 by one-way anova and Newman Keuls). CD107a was increased with IL-15 in NK cells but not in CD8 or NKT cells (Figure 2c). The gating strategy to investigate antitumour toxicity is shown in Supplementary Figure 1. The spontaneous background killing seen with PC3 and LNCaP cells alone is similar to the killing seen in the presence PBMCs with PBS. (Supplementary Figure 2)

To demonstrate that the expansion of NK cells in the human lymphocyte tumour cell cocultures was not due to an allogeneic response caused by a mismatch of MHC in the two populations, we repeated the NK expansion and tumour cell killing experiments in the murine syngeneic model where the TRAMP-C1 tumour cells are derived from C57/BL6 mice, which are also used for their splenocytes. In the murine syngeneic prostate tumour cell – splenocyte co-cultures (Figure 3), again, IL-15 led to expansion of NK cells in the co-cultures that was on average, five-fold greater than splenocytes incubated with IL-15 alone (n= 3 experiments, *p<0.05 by one-way anova and Newman Keuls). This effect also occurred with NKT cells, (n= 3 experiments, *p<0.05 by one-way anova and Newman Keuls), which was not seen in

the human co-culture model. Tumour cell killing of TRAMP-C1 cells was again only observed with IL-15.

Tumour cell killing in the co-cultures is mediated by NK cells and not CD8 T cells.

When co-cultures of PC-3 or LNCaP tumour cells were prepared with lymphocytes depleted of CD8 T cells, the expansion of NK cells and killing of the tumour cells remained similar to that with co-cultures containing non-depleted lymphocytes (Figure 4). When CD56⁺ cells were depleted, there was no increased killing seen higher than the tumour cell only control, therefore the tumour killing in the co-cultures is mediated by NK cells or NKT cells, and not CD8 T cells (n= 4 experiments for each condition). The efficacy of CD56⁺ and CD8 T cell removal is shown in dot plots in Supplementary Figure 3. Tumour cell killing in the murine syngeneic co-culture model is also mediated by NK cells (Supplementary Figure 4) as the killing by MHC class II depleted splenocytes and purified murine NK cells is similar and no killing is seen with murine purified CD8 T cells.

IL-15 modulates NKG2D, DNAM-1 and the NK ligands HLA-BW4, ULBP1 and Nectin 2 in both PC-3 and LNCaP co-cultures.

We wanted to examine whether the IL-15-mediated increased expansion of NK cells in the presence of PC-3 and LNCaP cells was associated with modifications in the expression of NK receptors on the NK cells, or NK receptor ligands on the tumour cells. Among the NK receptors studied (KIR2DL1, KIR2DL2, KIR3DL1, NKp30, NKp44, NKp46, DNAM-1, and NKG2D), only DNAM-1 and NKG2D were significantly upregulated by IL-15 (Figure 5) (by up to two-fold, n= 6, *p<0.05 by one-way anova and Newman Keuls) and therefore results

with KIR2DL1, KIR2DL2, KIR3DL1, NKp30, NKp44 and NKp46 are not shown. The gating strategy to measure NK receptor expression is shown in supplementary Figure 5. Among the NK ligands, HLA-BW4, ULBP1 and Nectin 2 were significantly inhibited in both PC-3 and LNCaP cells co-cultured with lymphocytes (Figure 6), however, MICA was strongly inhibited in LNCaP cells only. To determine whether IL-15 was inhibiting shed MICA, Nectin 2 and ULBP1, we performed ELISAs on the supernatants from the co-cultures. MICA shedding was decreased significantly in both PC-3 and LNCaP cells (by 50% and two-fold respectively- $n=4$, **** $p<0.0001$ by one-way anova and Newman Keuls post-hoc test), whereas there was no effect on Nectin 2 secretion (Figure 7), and ULBP1 was not detected (data not shown).

Inhibition of NKG2D does not prevent expansion of NK cells in the PC-3 or LNCaP – lymphocyte co-cultures.

When we performed blocking experiments on the lymphocyte-tumour cell co-cultures by adding a blocking antibody to NKG2D (or the isotype control) (Figure 8), there was no significant difference seen in the expansion of the NK cells in the co-cultures. Therefore NKG2D, although increased, does not play a role in the expansion of the NK cells.

Hsp90 and HSp70 are secreted from both cancerous (PC-3) and non-cancerous cells (PNT2, WPMY-1)

Although PC-3 secreted large amounts of HSP90 (>100 ng/ml), as seen by ELISA, there was little or no secretion by LNCaP cells and significant secretion by PNT2 cells (>20 ng/ml) (Figure 9). With HSP70, PNT2 cells secreted large amounts of the protein (>50 ng/ml)

whereas PC-3 and LNCaP had low levels of the protein in the supernatant (<15 ng/ml) and WPMY-1 had moderate levels (>20 ng/ml).

Discussion

There is a strong rationale that intratumorally targeted immunotherapeutics will be more effective and less toxic than those that are systemically administered [30, 31] and could also be used in late stage disease due to the abscopal phenomenon, where for example, IL-2 injected into melanoma lesions can clear distant metastases [32, 33]. In prostate cancer, intratumoural administration is easily possible due to the availability of MRI guided biopsy technologies where needles can deliver agents accurately to the lesion, in addition to taking biopsies [34].

In the present study we sought to determine whether cytokines with known NK and T cell activating properties would be able to activate these cells in the presence of prostate cancer cells, in order to identify cytokines that could be used intratumourally in prostate cancer patients. Some previous studies have shown expansion of NK cells with IL-15 using “feeder” leukemia cells but none have used prostate cancer cells [35]. Although an in-vitro co-culture system cannot compare with a complex in-vivo tumour microenvironment, the knowledge that prostate cancer cells exert much of the immunosuppressive effects on immune effector cells in this milieu [2] can give us an indication of cytokines that would be functional in this environment. Also, the use of metastatic prostate cancer cell lines PC3 and LNCaP, which are highly tumorigenic, enables us to identify cytokines that can work despite this extra degree of immunosuppression that is exerted, compared with primary prostate tumour cells. In the traditional in-vitro setting where cytokines are cultured with lymphocytes alone, IL-15

is a potent activator of NK, NKT cells and CD8 T cells. The cytokines IL-2, IL-12, IFN γ , and IL-21 can also activate NK cells and CD8 T cells: IL-2, IL-12 and IL-15 induce cytotoxic activity of NK cells toward K562 tumour cells to a similar extent [36]. However, in the present study only IL-15, among these cytokines, expands NK cells in the presence of prostate cancer or non-cancerous cells. The expanded cells are functional as both perforin and CD107a expression on NK cells are increased and cytotoxicity toward prostate cancer cells is also seen.

The action of IL-15 on effector cells in the presence of prostate cancer cells is not due to an alloreactivity of the effector cells toward the tumour cells as NK and CD8 T cells are also expanded within syngeneic co-cultures of murine C57/BL6 lymphocytes and TRAMP-C1 tumour cells in the presence of IL-15, but not other cytokines (as listed above).

Based on the lack of killing seen with CD56 depleted lymphocytes, and also with purified CD8 T cells in mice, the killing of prostate cancer cells in this model is due to NK/NKT cells. Therefore, although CD8 T cells are expanded and activated by IL-15, as expected these cells are not primed to kill tumour cells in an antigen dependent manner in this model as there are minimal numbers of APCs within the non-adherent lymphocytes.

NK cells are important in the anti-tumour immune response of Prostate Cancer patients: Overall survival (OS) and time to castration resistance (TCR) in metastatic prostate cancer patients are closely correlated with the efficacy of the NK cells (their ability to kill prostate cancer cells and high levels of activating receptors such as NKG2D) in these patients[37]. Furthermore, infiltration of NK cells into the cancerous prostates of men analyzed after prostatectomy correlated with a lower risk of progression after androgen deprivation therapy (ADT)[38].

A number of studies have examined adoptive transfer of NK cells for immunotherapy. These studies have had varying success depending on the types of tumour or whether NK cells were allogeneic or autologous: Studies using allogeneic NK cells have been successful in patients with melanoma, renal cell carcinoma, refractory Hodgkin's disease and refractory AML whereas use of autologous cells has proved less successful[39]. However, these studies have so far not used IL-15 as an expanding cytokine, due to its previously poor availability in GMP form. The use of IL-15 may be a game changer in these therapies[40].

Based on our finding that IL-15 increases NKG2D in NK cells co-cultured with tumour cells compared to lymphocytes cultured alone, and concomitantly decreases shed MICA, we posited that the interaction of NKG2D with its ligands on tumour cells may be at least a partial reason for the expansion of the NK cells in the co-cultures. Although the MICA binding to NKG2D is contact-based, it is known that some forms of shed ligands can activate NKG2D, at least in the mouse, and this may be occurring through as yet unknown ligands in the co-cultures where an insert is present [41]. Also, NKG2D is activated directly by IL-15 and its expression is increased, but further activation may be occurring because of the inhibition of MICA shedding.

When we block NKG2D in our co-cultures with a blocking antibody however, there is little or no effect on expansion of the NK cells, suggesting that other molecules from the tumours are increasing the expansion of NK cells in the presence of IL-15. We wanted to study whether this was related to the expression of molecules in tumorigenic cells that were not expressed in normal cells. One such group of molecules are heat shock proteins, (HSPs), molecules first identified as proteins up-regulated with elevated temperature. Recently it was observed. that whereas normal cells can secrete these molecules when stressed, tumour cells,

can constitutively secrete these molecules and also express them on the cell surface [20, 42-44]. We have studied the involvement of HSP90 and HSP70 in the IL-15 mediated expansion of NK cells due to previous studies reporting where it has been reported that HSP90 and HSP70 are critical for NK cell function[21] [22].

However, our ELISAs show that whereas HSP90 is secreted at high levels in PC-3 cells, there is almost no secretion in LNCaPs and significant expression in WPMYI cells and PNT2 cells.

Therefore, other soluble factors must be in play to contribute to the increased NK expansion. It is also possible that expansion of NK cells is enhanced due to the greater availability of IL-15 occurring in the presence of prostate cancer cells: In tissues expressing IL-15R alpha, IL-15 is retained due to cell endosomes internalizing but not degrading IL-15-IL-15 receptor alpha complexes[45], and we have shown that prostate cancer cells express IL-15 alpha receptor, although this has no effect on prostate cell proliferation[46].

In summary, IL-15 can expand NK cells within co-cultures of lymphocytes and tumour cells, unlike IL-2, IL-12, IL-21 or IFN γ . This suggests that IL-15 may be a potent immunotherapeutic agent when administered intratumourally in prostate cancer. We are therefore now patenting a novel localisable form of this agent, and a first-in man trial with intratumoural IL-15 is planned imminently.

Conflicts of interest

None disclosed

Figure Legends

Figure 1 : Expansion of immune effector cell populations in the presence or absence of prostate cancer cells or normal prostate cells (with or without contact with lymphocytes “membrane inserts”) treated with 25 ng/ml of IL-15, IL-2, IL-12, IFN- γ , IL-21 or PBS (control). Figure (1a) Gating strategy for NK cells showing the significant increase in CD56⁺CD3⁻ cells in PC3 and LNCaP co-cultures treated with IL-15 compared to lymphocytes treated with IL-15 alone. Figure (1b) Quantitated expression of NK, NKT, CD8 T cells and Treg effector cells in the co-cultures of prostate cells and lymphocytes compared to lymphocytes alone. Results are expressed as % of the expression of cells obtained for the control (PBS) cultures as the expression of the effector cells differed widely with different donors. Culture with tumorigenic cell lines PC-3 and LNCaP increases expansion of NK cells by IL-15 to a greater extent (up to 5.1 fold) than lymphocytes cultured with IL-15 alone (2.1 fold) (n= 6, ** p<0.05 by one-way anova and Newman Keuls post hoc tests) and occurs independently of tumour cell contact i.e. expansion is not significantly different in the presence or absence of a membrane insert placed in the well to separate lymphocytes and prostate cells. The non-tumorigenic cell lines WPMY-1 and PNT2 cocultured with lymphocytes and IL-15 have NK cell expansion to the same extent as in lymphocytes alone treated with IL-15. The cytokines IL-2 and IL-12 give a small expansion of NK cells in lymphocytes alone but no expansion occurs in the co-cultures with any cytokine except IL-15. NKT cell expansion with IL-15 is similar in co-cultures of all prostate cells with or without tumour cell contact and occurs to the same extent in lymphocytes cultured alone with IL-15 (up to a 4-fold increase). CD8 expansion is seen only with IL-15 in lymphocytes alone or in co-cultures (up to a 1.5 fold increase, n= 6, * p <0.05 by one-way anova and newman Keuls post-hoc analysis). There is no significant difference in CD8 expansion when a membrane insert is present in the wells, separating lymphocytes from prostate cells. The

analysis of Treg expression shows that IL-15 inhibits Tregs either with lymphocytes alone or in the co-cultures with prostate cells. Results are the means +SEM from 6 separate experiments (n= 6, * p < 0.05, ** p < 0.01 by one-way anova and Newman Keuls post-hoc analysis).

Figure 2: IL-15 treatment induces effector cell-mediated killing of PC-3 and LNCaP cells in lymphocyte-tumour cell co-cultures (Fig 2a). The killing of PC-3 and LNCaP is increased to a mean of 55 and 60% as measured by annexin+ PI+ cell expression, compared with 15-20% in the co-cultures incubated with PBS (this is the same background cell death seen in the irradiated tumour cells alone after 7 days) (n = 4 * p<0.05 by one-way anova and Newman Keuls post hoc test). IL-12 and IL-21 also increased the cell killing in PC-3 cells, but not LNCaP. This is concomitant with an increased perforin in the NK, NKT and CD8 T cells in IL15 treated co-cultures compared to the PBS control (n= 4, * = p < 0.05 by one-way anova and Newman Keuls post hoc test)(Figure 2b). CD107a was also increased by IL-15 in the NK cells, but not NKT or CD8 T cells (n= 4, * p < 0.05 by one-way anova and Newman Keuls post hoc test) (Figure 2c).

Figure 3: IL-15 increases NK, NKT and CD8 T cell expansion, and tumour cell killing in murine C57/BL6 syngeneic splenocyte: TRAMP-C1 co-cultures (Panels A, B, C and D respectively). Splenocytes were depleted of MHC class II expressing cells as described in the materials and methods. Co-cultures of these splenocytes were then set up at an 8:1 ratio with irradiated TRAMP-C1 cells for 9 days in the presence of either PBS or murine IL-2, IL-12, IFN- γ , IL-15 or IL-21, all at 25 ng/ml. Cells were harvested and effector cell expression was measured on the FACsCalibur™ as described in the materials and methods. Panels A-C show

that NK cells are expanded in IL-15 treated splenocytes alone and in the co-cultures, with NK cell expansion being significantly higher (five-fold) in the co-cultures compared to splenocytes alone with IL-15 ($n = 3$, $**p < 0.05$ by one-way anova and Newman Keuls post hoc test). NKT and CD8 T cells are also significantly expanded in IL-15 treated co-cultures, but not in the splenocytes cultured with IL-15 alone ($n = 3$, $*p < 0.05$ by one-way anova and Newman Keuls post hoc test). The presence of an insert separating splenocytes and tumour cells again does not affect expansion of the NK and NKT effector cells. Effector mediated cell killing of TRAMP-C1 cells (carried out as described in the materials and methods) also occurs only with IL-15 incubation in co-cultures (Fig. 3D).

Figure 4: PC-3 and LNCaP killing in the tumour cell-lymphocyte co-cultures is mediated by CD56+ cells and not CD8 T cells. Results are expressed as increases in killing of PC3 or LNCaP cells in co-cultures treated with IL-15 (black columns) compared to the control killing in co-cultures with the PBS control (grey columns) in the three preparations respectively ($n = 3$, $*p < 0.05$ by one-way anova and Newman Keuls post hoc test). Depleted lymphocytes had levels of CD8 or CD56+ cells that were less than 0.1% of the lymphocyte population. The level of the cell killing in the control co-cultures was between 15-20% as previously observed. Data was normalized so the control co-cultures with depleted and non-depleted populations of lymphocytes were set at 100%.

Figure 5. Effects of IL-15 on the expression of NK receptors NKG2D and DNAM-1. IL-15 treatment of lymphocytes increases NKG2D expression on NK cells compared to that in PBMCs treated with PBS or IL-2 ($n = 6$, $*p < 0.05$ by one-way anova and Newman Keuls post-hoc test) and the expression in co-cultures of lymphocytes with PC3 and LNCaP is

increased further compared to IL-15 treated lymphocytes alone (n= 6, ** p<0.01 by one-way anova and Newman Keuls post-hoc test). DNAM-1 expression is also increased with IL-15 to a similar extent in lymphocytes alone or with tumour cell co-cultures (n= 6, * p<0.05 by one-way anova and Newman Keuls post-hoc test). The addition of low dose IL-2 does not significantly inhibit the effects of IL-15 on these receptors.

Figure 6: Effects of IL-15 on the expression of NK ligands on PC-3 and LNCaP tumour cells in lymphocyte co-cultures or cultured without lymphocytes. IL-15 and IL-15 + low dose IL-2 inhibits the expression of the NK ligands ULBP-1, HLA-G and HLA-BW4 on both PC-3 and LNCaP cell lines co-cultured with lymphocytes. Results are expressed as the percentage of expression in PBS control PC-3 or LNCaP co-cultures or tumour cells alone (n= 6, * p<0.05 by one-way anova and Newman Keuls post-hoc test). MICA, Nectin 2 and HLA- Class I are inhibited by IL-15 and IL-15 + low dose IL-2 only in LNCaP cells co-cultured with lymphocytes (n= 6, * p<0.05 by one-way anova and Newman Keuls post-hoc test).

Figure 7: Effects of IL-15 on shedding of the NKG2D ligands MICA and Nectin 2: IL-15 and IL-15 plus 10 units/ml IL-2 inhibits shedding of MICA in PC-3 and LNCaP cells but not Nectin 2. In PC-3 cell cocultures with lymphocytes, MICA is lowered from a mean of 3000 pg/ml to 2500 pg/ml with IL-2 (both at 10 units/ml and 100 units/ml) but is lowered to a mean of 2000 pg/ml with IL-15 or IL-15 and low dose IL-2 (n= 4 ** p<0.05 with one-way anova and Newman Keuls post hoc test, **** p <0.001 with one-way anova and Newman Keuls post hoc test). In LNCaP co-cultures, shed MICA is lowered from an average of 400 pg/ml to an average of 100 pg/ml in both IL-15 and IL-15 + low dose IL-2, (n= 4, *** p <0.005 with one-way anova and Newman Keuls post hoc test).

Figure 8: Antibodies to NKG2D do not inhibit the increased expansion of NK cells by IL-15 in the presence of LNCaP (top panel) or PC-3 (bottom panel) cells. LNCaP or PC-3-lymphocyte cocultures set up for 7 days in the presence of 10 µg/ml blocking NKG2D antibody (R and D systems, UK) were harvested and NK cell expansion was measured by flow cytometry (BD FACsCalibur™). The expansion of NK cells was increased by IL-15 in lymphocytes cultured without tumour cells (n = 6, *p <0.05 by one-way anova and post hoc Newman Keuls test) and was greater in IL-15 treated lymphocyte co-cultures with PC-3 or LNCaP than with IL-15 treated lymphocytes only (n=6 **p <0.01 by one-way anova and post hoc Newman Keuls test) but NKG2D antibodies did not significantly affect expansion in these co-cultures.

Figure 9: HSP90 and HSP70 are secreted by both cancerous (PC-3 and LNCaP) and non-cancerous WPMY-1 and PNT2) cell lines. PC-3 secretion of HSP90 increases with IL-15 in lymphocyte co-cultures up to 100 ng/ml but LNCaP and WPMY-1 secretion of HSP90 is negligible (<10 ng/ml with any treatment. n= 3 experimental repeats). PNT2 secretion of HSP90 is moderate (>20 ng/ml, n= 3 experimental repeats). With HSP70, PNT2 cells secrete the largest amounts (>60 ng/ml, n= 3 experimental repeats) with low amounts secreted by PC-3, LNCaP, and WPMY-1. Therefore, there is no correlation between HSP90 or HSP70 secretion and tumorigenic or non-tumorigenic cells.

Supplementary Figure 1: Gating strategy and representative dot plots showing tumour cell cytotoxicity in lymphocyte: LNCaP cell co-cultures treated with PBS and IL-15 respectively as measured by Annexin IV and propidium iodide.

Supplementary Figure 2: Killing of PC3 and LNCaP tumour cells by non-adherent PBMCs. The graph shows that the background annexin/PI staining (representing cell death) in the PC3 and LNCaP cultures alone is similar to the percentage of staining with PC3 or LNCaP cells cocultured with non-adherent PBMCs with PBS or IL-2 (15-20%). Killing is increased to 50-60% in the co-cultures in the presence of IL-15. (n=3, * = $p < 0.05$, by one way anova and post hoc Newman Keuls test).

Supplementary Figure 3: Dot plots showing the lack of CD8 or CD56+ cells in lymphocytes depleted of these cells by the Miltenyi cell isolation kits for removal of CD8 or CD56+ cells.

Supplementary Figure 4: Killing of TRAMP-C1 cells in the MHC-class II depleted splenocyte -TRAMP_C1 co-cultures is mediated by NK cells and not CD8 T cells. The results are expressed as the percentage of dead TRAMP-C1 cells observed when TRAMP-C1 cells are co-cultured for 24 hrs with either MHC class II depleted splenocytes, purified NK cells or purified CD8 T cells that have been previously cultured for 9 days with TRAMP-C1 cells. N= 3 * $p < 0.05$ by one way anova and post hoc Newman Keuls tests. The ratios of cells used for the co-cultures were 8:1 (effector: target) for the MHC-Class II depleted splenocytes, 4:1 for the NK cells and 4:1 for the CD8 T cells.

Supplementary Figure 5: Gating strategy to measure NK cell receptors in NK cells in the lymphocytes alone or lymphocyte: tumour cell co-cultures. The lymphocyte gate is selected from the FSC vs SSC plot and then this gate is used to select CD56+ and CD3 negative cells

(Top left quadrant gate in middle dot plot). This second gate is then used to plot FL1 (which measures the NK cell receptors as these are all FITC conjugated antibodies), and FL3 (which is the CD8 –PerCP channel in this experiment and should be at background level as NK cells are not normally CD8+). Receptor expression is then taken from the bottom right quadrant which measures NK cells with the receptor, e.g NKG2D or KIR2DL1.

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